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# Effects of vascular endothelial growth factor on endothelin-1 production by human lung microvascular endothelial cells in vitro

Gregory P. Star<sup>a</sup>, Michele Giovinazzo<sup>a</sup>, Esther Lamoureux<sup>b,†</sup>, David Langleben<sup>a,\*</sup><sup>a</sup> Lady Davis Research Institute and Center for Pulmonary Vascular Disease, Division of Cardiology, Jewish General Hospital, McGill University, Montreal, Quebec, Canada<sup>b</sup> Department of Pathology, Jewish General Hospital, McGill University, Montreal, Quebec, Canada

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## ABSTRACT

**Aims:** Increased endothelin-1 (ET-1) is a hallmark of pulmonary arterial hypertension (PAH), and contributes to its pathogenesis. The factors controlling ET-1 in PAH are poorly understood. Combined with other stimuli, vascular endothelial growth factor (VEGF) blockade results in PAH-like lesions in animal models, and has been associated with PAH in humans. The effects of VEGF on ET-1 production by human lung blood microvascular endothelial cells (HMVEC-LBI) are unknown.

**Main methods:** We exposed HMVEC-LBI in-vitro to human VEGF-121 (40 ng/mL) in serum-free medium for 7 h, in the absence or presence of the VEGF receptor antagonist, SU5416 (3 and 10  $\mu$ M). ET-1 production was measured in the supernatant. Phosphorylation of VEGF receptor 2 (VEGFR2) was measured by Western blotting after exposure to VEGF without or with SU5416 for 5 and 10 min.

**Key findings:** VEGF effectively caused VEGFR2 phosphorylation, which was blocked by SU5416. VEGF decreased ET-1 production by at least 29%. In the absence of VEGF, SU5416 increased ET-1 production, by 16% at 10  $\mu$ M, and SU5416 was able to completely abolish the VEGF effect on ET-1 production.

**Significance:** VEGF may promote vascular health by decreasing ET-1 production in HMVEC-LBI. Blockade of VEGF signaling by SU5416 increases ET-1 levels. The role of VEGF in modulating endothelin production in PAH deserves further study.

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## Introduction

The pathogenesis of pulmonary arterial hypertension (PAH) involves microvascular endothelial proliferation and dysfunction, with formation of plexiform lesions (Morrell et al., 2009; Tuder et al., 2009). One manifestation of this dysfunction is increased production of the deleterious vasoconstrictor and mitogen, endothelin-1 (ET-1) (Giaid et al., 1993; Stewart et al., 1991). It has been proposed that in PAH a variety of injurious stimuli result in endothelial apoptosis, with emergence of apoptosis-resistant endothelial clones that narrow the vascular lumen, restricting blood flow and ultimately causing right heart failure and death (Teichert-Kuliszewska et al., 2006; Tuder et al., 2001a).

Vascular endothelial growth factor (VEGF) is important in endothelial homeostasis, controlling differentiation, mitogenicity and

endothelial survival (Ferrara, 2004). VEGF increases nitric oxide and prostacyclin production. Loss of VEGF signaling results in endothelial apoptosis. However, the role of VEGF in PAH is complex. Levels of VEGF and its receptors are increased in PAH (Hirose et al., 2000; Tuder et al., 2001a). Confounding the issue is the finding that VEGF blockade in animals, combined with other stimuli such as hypoxia, causes pulmonary hypertension and has provided a useful model of PAH (Taraseviciene-Stewart et al., 2002; Tuder et al., 2001b). Loss of VEGF might contribute to the endothelial apoptosis that is thought to be an initiating event in PAH (Teichert-Kuliszewska et al., 2006). Indeed, PAH has been reported in humans after treatment with bevacizumab, a VEGF blocker (Liotta et al., 2009). Thus, VEGF may play a role in the pathogenesis of PAH.

The stimuli for increased ET-1 in PAH are unknown. It is not known how ET-1 synthesis by pulmonary microvascular endothelium is affected by VEGF. We therefore explored the effects of VEGF on ET-1 production. The bone morphogenic protein (BMP) and transforming growth factor- $\beta$  (TGF $\beta$ ) receptor pathways are highly involved in the development of heritable PAH (Morrell et al., 2009). BMP-9 is a strong activator of endothelial activin receptor-like kinase 1 (David et al., 2007). We have previously demonstrated that BMP-9 stimulates ET-1 production by lung microvascular endothelial cells in vitro (Star et al., 2010). We

\* Corresponding author at: Room E-206, Jewish General Hospital, 3755 Cote Ste Catherine, Montreal, Quebec H3T 1E2, Canada. Tel.: +1 514 340 7531; fax: +1 514 340 7534.

E-mail address: [david.langleben@mcgill.ca](mailto:david.langleben@mcgill.ca) (D. Langleben).

<sup>†</sup> Deceased.

therefore also studied the interactions of BMP9 and VEGF, in terms of effects on ET-1 production.

## Materials and methods

### Cell cultures

Human microvascular endothelial cells of the lung-blood (HMVEC-LBI, Lonza, Walkersville, MD) or human umbilical vein endothelial cells (HUVEC, Lonza, Walkersville, MD) were plated into 24-well plates and grown to confluence in EGM-2MV medium (Lonza) containing 2.5% fetal bovine serum (FBS) plus supplements. Only cells in passage 5 were used, with  $n = 12$  wells per experimental condition.

### Experimental design

To confirm in HMVEC-LBI the appropriate signaling of VEGF via its receptor, phosphorylation of the VEGF receptor 2 (VEGFR2) was measured by Western blotting after exposure to VEGF-121 (0 or 40 ng/mL, Peprotech, Rocky Hill, NJ) in serum-free medium with 0.1% bovine serum albumin (BSA), with or without the VEGF receptor antagonists, SU5416 (3  $\mu$ M, CID 5329098, Cayman Chemical, Ann Arbor, Michigan) or (E)-FeCP-oxindole (1  $\mu$ M, CID 57369962, Tocris, Minneapolis, MN), for 5 and 10 min. The cells were then immediately lysed in SDS sample buffer. The cell lysates were separated on a polyacrylamide gel and then transferred to a PVDF membrane. Afterwards, the samples were blocked in 5% fat free milk in TBST for 1 h. The membranes were subsequently incubated overnight at 4 °C in TBST with 5% BSA containing antibodies to either rabbit anti-P-VEGFR2 (Cell Signaling, Danvers, MA) or the housekeeping gene mouse anti-GAPDH (Fitzgerald, Concord, MA). All membranes were then washed in TBST and incubated with anti-rabbit (Cell Signaling, Danvers, MA) or anti-mouse (Abcam, Cambridge, UK) antibodies. After a final washing the membranes were incubated with Western Lightning Plus-ECL (Perkin Elmer, Waltham, MA) and exposed to film.

To study the effects on ET-1 production and to search for a dose response, after confluence of HMVEC-LBI or HUVEC, the medium was replaced with medium containing VEGF-121 (0–40 ng/mL) in serum-free medium for 7 h. The supernatants were collected at the end of the study period, and frozen at  $-70$  °C. To examine interactions between VEGF and BMP9 with reference to ET-1 production, the HMVEC-LBI and HUVEC were exposed to VEGF-121 (40 ng/mL) and/or BMP9 (2.5 ng/mL, R&D Systems, Minneapolis, MN) in serum-free medium for 7 h. The supernatants were then collected and frozen as above. In another experiment, HMVEC-LBI were exposed to VEGF-121 (40 ng/mL) in serum-free medium for 7 h, with or without the VEGF receptor antagonist SU5416 (0–10  $\mu$ M). The supernatant was collected and frozen. In all these experiments, the supernatant was subsequently thawed and immunoreactive ET-1 levels were measured by ELISA (Enzo Life Sciences, Ann Arbor, Michigan) and expressed as pg/mL.

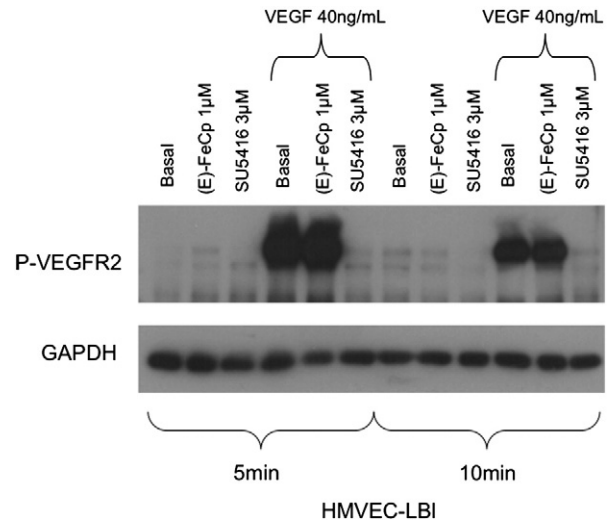
### Statistics

Data are expressed as mean  $\pm$  SD. To compare groups, analysis-of-variance was performed and followed, where appropriate by the Tukey–Kramer multiple comparison test.

## Results

### Effects of VEGF and its blockers on VEGF receptor phosphorylation (Fig. 1)

Using Western blotting, in the HMVEC-LBI and in serum-free medium, there was no evidence of background VEGF receptor-2 phosphorylation. Addition of the VEGF receptor blockers, (E)-FeCP-oxindole and SU5416, did not affect the background phosphorylation.



**Fig. 1.** Western blot showing levels of phosphorylated VEGF receptor-2 (P-VEGFR2), in HMVEC-LBI in basal medium and after 5 and 10 minute exposure to VEGF (40 ng/mL). The VEGF receptor blockers (E)-FeCP-oxindole and SU5416 were added in the indicated lanes. GAPDH is used as a loading control.

Addition of VEGF caused rapid phosphorylation as detected after 5 min, and this was sustained although slightly diminished at 10 min. Attempted blockade with (E)-FeCP-oxindole at the manufacturer's recommended dose had no effect on the phosphorylation. However, SU5416 blocked the effects of VEGF, completely preventing VEGFR2 phosphorylation.

### Effects of VEGF on ET-1 production (Fig. 2)

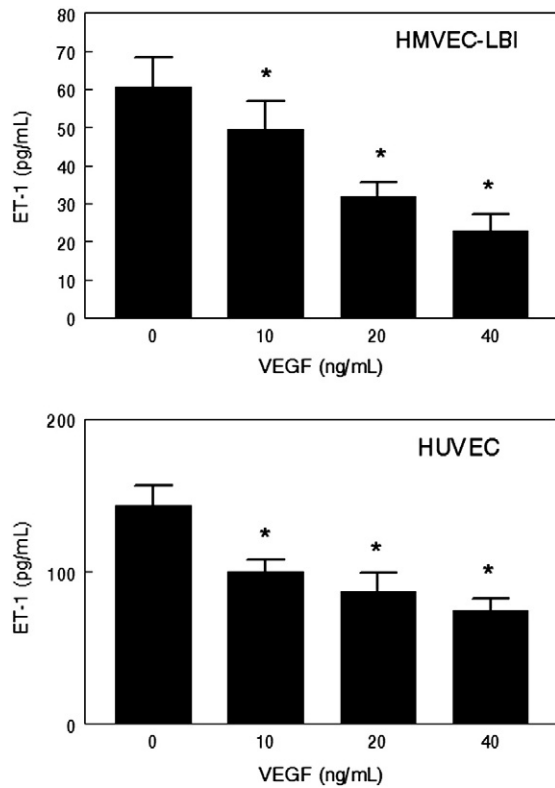
In both HMVEC-LBI and HUVEC, exposure to VEGF significantly decreased ET-1 production, as assessed by ET-1 levels in the supernatant, after 7 h. This reduction occurred in a concentration-dependent manner, with the greatest effect in each case seen at VEGF 40 ng/mL. At that concentration, ET-1 levels were decreased by 63% in HMVEC-LBI and by 48% in HUVEC. Within the range studied, the dose response was more evident in HMVEC-LBI.

### Effects of VEGF receptor blockade on ET-1 production (Fig. 3)

In HMVEC-LBI not exposed to VEGF, and in serum-free medium, addition of the VEGF receptor blocker, SU5416, significantly increased basal ET-1 production, by up to 21% at 3 ng/mL,  $p < 0.02$ . Addition of VEGF alone significantly decreased ET-1 production by 29% versus no VEGF,  $p < 0.02$ . In the presence of VEGF, addition of SU5416 completely blocked the inhibitory effects of VEGF on ET-1 production, with an increase in ET-1 levels of 82% as compared to VEGF alone. Furthermore, in the presence of SU5416, the ET-1 levels even surpassed those of the baseline control (no VEGF) by 30%,  $p < 0.02$ . At 3 ng/mL of SU5416, the ET-1 levels in the presence of VEGF were also slightly higher than those with SU5416 (3 ng/mL) in the absence of VEGF,  $p < 0.02$ .

### Effects of VEGF and BMP-9 on ET-1 production (Fig. 4)

In HMVEC-LBI, VEGF (40 ng/mL) significantly reduced ET-1 production by 29%. As has been previously described, addition of BMP-9 (2.5 ng/mL) greatly stimulated ET-1 production, by 81% over baseline. Combination of VEGF and BMP-9 resulted in lower ET-1 levels as compared to BMP-9 alone, but they were still 35% higher than baseline and 90% higher than with VEGF alone. A similar pattern was observed when HUVEC were studied.

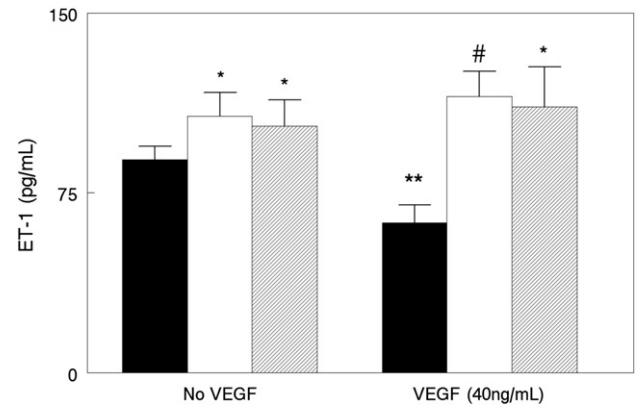


**Fig. 2.** Effects of various concentrations of VEGF on ET-1 levels in the supernatant of HMVEC-LBI (upper) and HUVEC (lower) after a 7 h exposure. Bars are mean + SD. \*,  $p < 0.02$  versus no VEGF.

## Discussion

The available data on VEGF and PAH might seem paradoxical, but it is clear that VEGF and its absence are implicated in the life and death of pulmonary microvascular endothelial cells in PAH, and in endothelial dysfunction (Voelkel et al., 2002). Levels of both VEGF and its receptor are increased in PAH, but it is unclear if they are protective, adaptive or a manifestation of cellular dysfunction (Geiger et al., 2000; Hirose et al., 2000; Niimi et al., 2000; Tudor et al., 2001a). VEGF therapy can attenuate pulmonary hypertension in animal models (Farkas et al., 2009). Although VEGF is a potent survival factor for endothelium, PAH is characterized by endothelial cell apoptosis and emergence of apoptosis resistant clones (Voelkel et al., 1998). Moreover, VEGF receptor blockade combined with other stimuli such a hypoxia or shear stress, induces PAH-like lesions in animals. VEGF receptor blockade by itself has been associated with PAH in humans receiving it for cancer therapy (Ciucan et al., 2011; Liotta et al., 2009). Thus, whether VEGF levels in established PAH support the remaining healthy endothelium, or are contributing to the excessive aberrant endothelial proliferation, is unknown.

VEGF promotes the production of mediators that are beneficial to vascular health, including nitric oxide and prostacyclin (He et al., 1999; Ku et al., 1993; Tsurumi et al., 1997). However, in patients with PAH, levels of nitric oxide and prostacyclin are reduced, and levels of the vasoconstrictor and vascular mitogen, ET-1, are increased (Christman et al., 1992; Giaid and Saleh, 1995; Giaid et al., 1993; Stewart et al., 1991; Tudor et al., 1999). We previously hypothesized that pulmonary microvascular endothelial dysfunction leads to increased ET-1 production in PAH and that this might be driven in part by mediator effects or altered gene expression in the cells. Indeed, knockdown of BMPR-2 in vitro, simulating heritable PAH, increases ET-1 production by human lung microvascular endothelial cells (Star et al., 2013). Likewise, exposure of the cells to BMP-9,

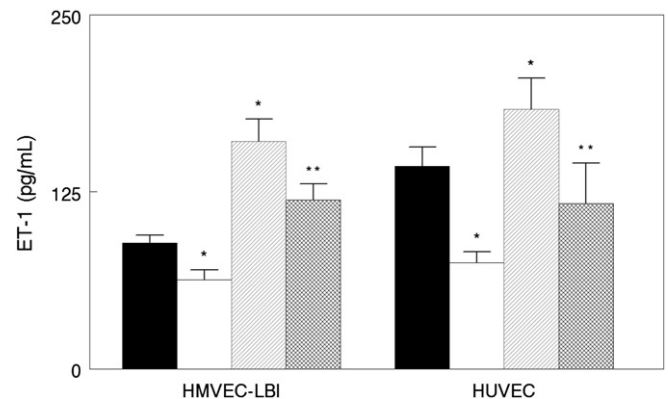


**Fig. 3.** Effects of the VEGF receptor blocker SU5416 0 ng/mL (control, black bars), 3 ng/mL (white bars) and 10 ng/mL (diagonal bars) on ET-1 levels in the supernatant of HMVEC-LBI in the absence or presence of VEGF (40 ng/mL), after a 7 hour exposure. Bars are mean + SD. \*,  $p < 0.02$  versus respective control; \*\*,  $p < 0.02$  versus other control; #,  $p < 0.02$  versus both controls and other 3 ng/mL group.

which activates a BMPR2 and ALK-1 complex, increases ET-1 levels (Star et al., 2010). Given the important effects of VEGF, potentially beneficial and detrimental, in PAH, we studied its effects on endothelin production.

Because of phenotypic heterogeneity between systemic and pulmonary endothelium, between macrovascular and microvascular endothelium, and between human endothelium and that of other animals, we performed all experiments using HMVEC-LBI, endothelial cells derived from human pulmonary microvasculature and therefore the cells most relevant to PAH. We first demonstrated that the cells had type 2 VEGF receptors that could be rapidly phosphorylated by exposure to VEGF. We demonstrated that the VEGF receptor antagonist SU5416 completely prevented this phosphorylation. We also tested (E)-FeCp-oxindole, which is purported to block VEGFR2 (Spencer et al., 2011), but it was ineffective at preventing VEGF-induced VEGFR2 phosphorylation, at doses well above the IC50 suggested by the manufacturer. Thus, in subsequent experiments only SU5416 was used. We cannot explain the lack of effect of (E)-FeCp-oxindole other than to say that it was consistent in our model.

We found that VEGF decreased ET-1 production by HMVEC-LBI, in a dose-dependent fashion. Although the effectiveness of VEGF varied between experiments, it consistently had an inhibitory effect. We also studied HUVEC and found a similar pattern, although the dose response



**Fig. 4.** Effects of VEGF (40 ng/mL) and BMP-9 (2.5 ng/mL) on ET-1 levels in the supernatant of HMVEC-LBI and HUVEC after a 7 hour exposure. Black bars are control, white bars are VEGF alone, diagonal bars are BMP-9 alone and hatched bars are VEGF and BMP-9 combined. Bars are mean + SD. \*,  $p < 0.02$  versus respective control; \*\*,  $p < 0.02$  versus all other respective groups for a given cell type.



was more evident with HMVEC-LBI. Our finding of an inhibitory effect of VEGF on ET-1 production by HUVEC differs from a previous study using HUVEC, where VEGF165, up to a dose of 10 ng/mL increased ET-1 production (Lee et al., 2007). There may be differences between VEGF 121 and VEGF 165 that are as yet untested, or the culture conditions may have been different. In another study, using bovine aortic endothelial cells, VEGF increased ET-1 secretion in vitro (Matsuura et al., 1998). We believe that we were seeing a VEGF-specific effect in reducing ET-1 production by the HMVEC-LBI cells because addition of SU5416 completely blocked the VEGF-induced decrease in ET-1. Moreover, SU5416 in the presence of VEGF increased ET-1 production to levels that exceeded the baseline control. In support of our findings, SU5416 increased circulating ET-1 levels in a structurally relevant murine model of PAH (Ciucan et al., 2011).

We previously showed that BMP-9 stimulates ET-1 production by HMVEC-LBI (Star et al., 2010). This result was reproduced in the present study. However, in both HMVEC-LBI and HUVEC, VEGF was able to reduce the BMP-9-induced stimulation of ET-1 production to a degree similar to its ability to decrease ET-1 production in cells not stimulated by BMP-9. Nevertheless, the ET-1 levels were never reduced by BMP-9 back to control levels of ET-1. An interaction between VEGF and ALK-1 has been described, and might provide a mechanism whereby VEGF competes against BMP-9 for ALK-1 binding (Hu-Lowe et al., 2011). In PAH, the endothelial cells are subjected to a multitude of mediators. Our result with BMP-9 suggests that in PAH, where VEGF levels are high and might inhibit ET-1 production, other cell signaling activators such as BMPs might overcome the inhibition and result in high levels of ET-1. Further study is needed in this area.

## Conclusion

VEGF reduces ET-1 production by HMVEC-LBI in vitro. This can be overcome by VEGF receptor blockade and by BMP-9, which interacts with BMPR2 and ALK-1. These are observational studies. The role of VEGF in modulating ET-1 levels in PAH, and the underlying mechanisms, deserve further study.

## Conflict of interest statement

The authors state that there are no conflicts of interest.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.lfs.2014.02.032>.

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